# Interaction of *Bartonella henselae* with Endothelial Cells Promotes Monocyte/Macrophage Chemoattractant Protein 1 Gene Expression and Protein Production and Triggers Monocyte Migration

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**Bacillary angiomatosis (BA), one of the many clinical manifestations resulting from infection with the facultative intracellular bacterium *Bartonella henselae*, is characterized by angiogenic lesions. Macrophages have been identified as important effector cells contributing to the angiogenic process during *B. henselae* infection by infiltrating BA lesions and secreting vascular endothelial growth factor. Monocyte-macrophage chemoattractant protein 1 (MCP-1) recruits macrophages to sites of inflammation. In this study, we investigated the ability of *B. henselae* to upregulate MCP-1 gene expression and protein production in the human microvascular endothelial cell line HMEC-1. MCP-1 mRNA was induced at 6 and 24 h after treatment with bacteria, whereas protein production was elevated at 6, 24, and 48 h. This induction was not dependent on the presence of bacterial lipopolysaccharide or endothelial cell toll-like receptor 4. However, MCP-1 production was dependent on NF-κB activity. Outer membrane proteins of low molecular weight were able to upregulate MCP-1 production. Furthermore, supernatants from *B. henselae*-infected HMEC-1 were able to induce chemotaxis of THP-1 monocytes. These data suggest a mechanism by which the macrophage effector cell is recruited to the endothelium during *B. henselae* infection and then contributes to bacterial-induced angiogenesis.**

*Bartonella henselae* is a fastidious gram-negative bacterial human pathogen of which the domestic cat is the natural reservoir. *B. henselae* can cause cat scratch disease, bacillary angiomatosis (BA), bacillary peliosis, bacteremia, and endocarditis (1, 3, 39, 48–50). BA is an opportunistic infection characterized by fever and angioproliferative lesions that may resemble Kaposi's sarcoma. Histological examination of BA lesions has revealed infiltration of polymorphonuclear leukocytes and macrophages (26, 27, 35). The exact mechanism of angiogenesis in BA is unknown; however, it has been shown that *B. henselae*-infected macrophages produce the pro-angiogenic mediator vascular endothelial growth factor (VEGF) (41). This VEGF production may be a crucial feature in the endothelial cell proliferation that is a hallmark of BA lesions. A paracrine loop has been proposed (41) in which the infected macrophage effector cell secretes VEGF and interleukin-8 (IL-8), which act as endothelial cell mitogens. In addition, the infected endothelial cell may upregulate expression and production of pro-angiogenic proteins such as chemokines, adhesion molecules (16), and cytokines (21, 41), while downregulating inducers of apoptosis such as caspases (22).

Pathogenic (tumor or inflammatory) angiogenesis provides a survival mechanism for the tissues of tumors and tumor-like lesions. The process of angiogenesis requires cooperation between cells, cytokines, growth factors, and matrix components. A sensitive balance between angiostatic and angiogenic factors must exist in order to control angiogenic activity; in tumors and tumor-like lesions, however, this tightly regulated system is upset (19). Angiogenesis is associated with conditions that involve inflammatory cell infiltrate (18), such as cancer, papopavirus infection, and herpesvirus infection (5, 7, 15, 29, 33). Angiogenesis and inflammation coordinate through common stimuli for endothelial cells and leukocytes; these stimuli include chemokines.

Angiogenic chemokines exert a direct effect on the endothelium and an indirect effect on angiogenic factor-expressing leukocytes (37, 52). Chemokines are induced by tumor necrosis factor (TNF) or IL-1 or by interaction with bacterial pathogens. Chemokines recruit leukocytes to sites of inflammation. Specifically, monocyte-macrophage chemoattractant protein 1 (MCP-1) stimulates chemotaxis of monocytes and macrophages to sites of inflammation (28). MCP-1 can also directly promote angiogenesis (37, 52). Thus, MCP-1 could play dual roles in the potential paracrine loop model of *B. henselae*-induced angiogenesis by acting in an autocrine-like manner on endothelial cells to promote angiogenesis, while recruiting macrophages, the effector cell in the model, to the site of infection.

Endothelial cells are critical for inflammation. They control vascular permeability, regulate coagulation and thrombosis, direct passage of leukocytes into areas of inflammation, and produce pro-inflammatory cytokines such as IL-1, IL-6, IL-8, and adhesion molecules. Vascular endothelial cells line the luminal side of blood vessels and mediate interactions between

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blood and tissue (10, 23). Although they are not professional phagocytes, endothelial cells express toll-like receptors (tlr) which can be activated in response to pattern recognition molecules and therefore play a role in innate immunity. Human microvascular endothelial cells (HMEC-1) express tlr4, which recognizes lipopolysaccharide (LPS), endogenous HSP60, chlamydial HSP60, and other lipoproteins (17, 25, 38, 44, 51). Toll-like receptor signaling results in activation of NF-κB and transcription of genes such as chemokines and inflammatory cytokines. Since chemokines are important for angiogenesis and macrophage recruitment, toll-like receptor signaling in endothelial cells could contribute to *B. henselae*-induced angiogenesis.

In order to better understand the response of endothelial cells to the pathogen *B. henselae*, we examined the effect of *B. henselae* infection on expression and protein levels of the chemokine MCP-1 in HMEC-1. In addition, we examined the ability of conditioned media from *B. henselae*-infected HMEC-1 to cause chemotaxis of THP-1 monocytes. The data support a role for MCP-1 during *B. henselae* infection whereby MCP-1 mediates macrophage infiltration, leading to an angiogenic state.

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## MATERIALS AND METHODS

**Bacterial strains.** *Bartonella henselae* Houston-1 (ATCC 49882) (40) strain was grown on chocolate agar prepared with heart infusion agar base (Difco, Detroit, MI) supplemented with 1% bovine hemoglobin (Becton Dickinson, Cockeysville, MD). Bacterial cultures were maintained at 37°C and 5% $CO_2$ and humidity to saturation. For certain experiments, bacteria were heat killed at 100°C for 30 min as described previously (41). *Escherichia coli* strain JM109 was grown in Luria-Bertani broth or agar (Difco).

**Cell lines.** The immortalized human microvascular cell line HMEC-1 (2) was cultured in MCDB131 cell culture media (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum (HyClone Laboratories, Logan, UT), 10 ng/ml epidermal growth factor, 1.461 g/liter L-glutamine, 1 μg/ml hydrocortisone, 50 μg/ml penicillin-streptomycin, 2.5 μg/ml amphotericin B (Sigma-Aldrich, St. Louis, MO), 2 mg/ml sodium bicarbonate, and 10 mM HEPES (Mediatech, Herndon, VA). Human THP-1 monocytes (54) were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal calf serum, 5 μM 2-mercaptoethanol (Sigma-Aldrich), 10 μg/ml vancomycin (Sigma-Aldrich), and 1 μg/ml amphotericin B. Cells were subcultured every 2 days and were used in experiments at passages from 6 to 10. Cell cultures were maintained at 37°C and 5% $CO_2$ and humidity to saturation.

**Coculture of endothelial cells and bacteria.** Prior to infection, the culture medium containing antibiotics was removed from cell cultures and replaced with MCDB131 without antibiotics or growth factors. Cells were permitted to adapt overnight. *B. henselae* Houston-1 cells were harvested from chocolate agar and suspended in cell culture media, and then the concentration of bacteria was determined spectrophotometrically. We have previously determined by plate counts that at an optical density of 600 nm, a reading of 0.5 is estimated to represent $10^9$ bacteria. *B. henselae* bacteria were added to cells at a multiplicity of infection of 500 unless otherwise indicated. Cells were cocultured with Houston-1 cells at various time points.

For *E. coli* cocultures, JM109 bacteria were harvested into cell culture medium and added to cells at a density of 100 *E. coli* cells per HMEC-1 cell, also determined spectrophotometrically (43). During the experiments with longer time courses and during inhibition assays, *E. coli* LPS (Sigma-Aldrich) was used as a positive control for MCP-1 production in order to keep HMEC-1 cells alive, as *E. coli* JM109 infection was found to be cytotoxic for HMEC-1 cells at later time points.

**Inhibition assays.** Polymyxin B sulfate (Sigma-Aldrich) was used to neutralize the bacterial LPS in some experiments. Houston-1 or *E. coli* JM109 cells or purified *E. coli* LPS was incubated with 30 μg/ml polymyxin B sulfate for 1 h at 37°C and 5% $CO_2$ before being added to HMEC-1. In some experiments, HMEC-1 cells were preincubated with a mouse monoclonal antibody (HTA 125) specific for human toll-like receptor-4 (tlr-4) (e-Bioscience, San Diego, CA) in order to determine whether tlr-4 signaling was required for MCP-1 production. HMEC-1 cells were preincubated with 20 μg/ml anti-tlr4 for 1 h at 37°C with gentle shaking, and then cocultures proceeded as usual and supernatants were collected 8 h after addition of bacteria or LPS. Live *E. coli* JM109 cells were cultured with HMEC-1 during some experiments in order to ensure that the tlr4 monoclonal antibody would block activity from the LPS of live *E. coli* as well as from purified LPS. An isotype control (mouse immunoglobulin G2a, κ [IgG2a, κ]) (e-Bioscience) was used as a control antibody at 20 μg/ml. During NF-κB inhibition assays, HMEC-1 cells were incubated with 50 μM pyrrolidinedithiocarbamate (PDTC) (Sigma) or 100 μM N-tosyl-L-phenylalanine (TPCK) (Sigma) for 1 h at 37°C with 5% $CO_2$ before infections. Inhibitors were maintained throughout the course of the assays.

**Semiquantitative RT-PCR.** Reverse transcription-PCR (RT-PCR) was performed on HMEC-1 cocultured with *B. henselae* or *E. coli* at the indicated times after infection. Total RNA was extracted with TRIzol reagent (Sigma-Aldrich) according to the manufacturer's protocol. Total RNA was treated with RNase-free DNase (Ambion, Inc., Austin, TX) according to the manufacturer's instructions. The concentration of RNA was determined spectrophotometrically using a GeneQuant II system (Pharmacia Biotech, Cambridge, England). cDNA preparation and subsequent PCR amplification were carried out with a One-Step RT-PCR kit (QIAGEN, Inc., Valencia, CA) in the presence of gene-specific primers and 2 μg total RNA. The PCR conditions were 1 min at 95°C, 1 min at 58°C, and 1 min at 72°C for 35 cycles. Primer sequences for RT-PCR were as follows: for β-actin forward, 5′-AGAAAATCTGGCACCACACC-3′; for β-actin reverse, 5′-CCATCTCTTGCTCGAAGTCC-3′; for MCP-1 forward, 5′-TTCTC AAACTGAAGCTCGCACTCTCGCC-3′; and for MCP-1 reverse, 5′-TGTGG AGTGAGTGTTCAAGTCTTGGGAGTT-3′. PCR products were analyzed by electrophoresis through 2% agarose gels and were visualized by ethidium bromide staining. Amplicon sizes were 434 bp and 348 bp for β-actin and MCP-1 primers, respectively. RT-PCR data were analyzed by scanning densitometry of gel bands with Kodak 1D Image Analysis software and normalizing to β-actin signals obtained from the same time points. RT-PCRs included a no-template control and a no-reverse-transcriptase control to exclude DNA or RNA contamination.

**MCP-1 ELISA.** MCP-1 levels in HMEC-1 supernatants were assayed with an MCP-1 DuoSet enzyme-linked immunosorbent assay (ELISA) development system (R & D Systems, Minneapolis, MN) according to the manufacturer's protocol. A 3,3′,5,5′-tetramethylbenzidine liquid substrate system (Sigma-Aldrich) was added, and the color was allowed to develop for 20 min. The reaction was stopped with 2 N sulfuric acid. ELISA plates were analyzed using a μQuant plate reader (Bio-Tek, Winooski, VT) at 450 nm.

**Chemotaxis of THP-1 monocytes.** Chemotaxis of THP-1 monocytes was examined with modified Boyden chambers (Neuroprobe, Cabin John, MD) according to the manufacturer's instructions. Briefly, the lower well of the chamber was filled with 1.2 ml supernatants from untreated, *B. henselae*-stimulated, or *E. coli*-stimulated HMEC-1. A total 510 μl of THP-1 cell suspension ($5 \times 10^5$ cells) was added to the upper well. A 5-μm-pore-size polyvinylpyrrolidone-free polycarbonate membrane (Neuroprobe) separated the two wells. Migration occurred while incubating the chambers at 37°C and 5% $CO_2$ with humidity for 4 h. After 4 h, the upper side of the membrane was scraped three times with a sterile swab soaked in phosphate-buffered saline to remove nonmigrated cells, and the lower side of the membrane was fixed and stained with Hema-3 Stat Pack (Biochemical Sciences, Inc., Swedesboro, NJ) according to the manufacturer's instructions. Cells were counted in five high-powered fields per membrane, and these numbers were averaged. Cell counts ranged from 6 to 10 cells per high-powered field, with the exception of cells that migrated in response to untreated HMEC-1 supernatants, in which case zero to two cells were counted per microscope field as a result of reduced migration of THP-1 cells. Results are expressed as a chemotactic index, in which the average number of cells that migrated in response to untreated HMEC-1 supernatants was set at 1. A graph of the means of chemotactic indices for three experiments is shown.

**Preparation of crude *B. henselae* LPS.** *B. henselae* LPS was isolated as described previously with modifications (56). Briefly, 3-day-old bacteria were harvested from chocolate agar and suspended in phosphate-buffered saline. The bacterial pellet was washed three times with sterile water. Bacteria were lysed with lysis buffer (6% sodium dodecyl sulfate [SDS], 60 mM Tris, 6% β-mercaptoethanol, 10 mM dithiothreitol) at 100°C. Proteins were digested by proteinase K treatment. Crude LPS was dialyzed against sterile endotoxin-free water for 4 days. These crude extracts were concentrated with Centricon YM-3 (Millipore Corp., Bedford, MA). Crude *B. henselae* LPS was separated by Tricine–SDS-

polyacrylamide gel electrophoresis and visualized by silver staining as described previously (56, 58). No proteins were visualized upon Coomassie blue staining. The concentration of *B. henselae* LPS was determined by separating both *B. henselae* and *E. coli* LPS (1 mg/ml) by SDS-polyacrylamide gel electrophoresis and silver staining and then scanning the gel and determining the concentration by densitometry.

**Enrichment of outer membrane proteins from *B. henselae* and fractionation by molecular weight.** *B. henselae* outer membrane proteins (OMPs) were enriched as previously described (6, 14). The sarkosyl-insoluble pellet was resuspended in 10 mM HEPES (pH 7.4). Protein concentrations were determined using the Lowry protein assay (30). Protein (600 μg) in 400 μl Laemmli sample buffer with 142 mM 2-mercaptoethanol was heated at 95°C for 5 min and separated in a single large well of a two-dimensional 4 to 12% Bis-Tris NuPAGE gel (Invitrogen, Carlsbad, CA). Four sections of the gel were excised corresponding to 3 to 33 kDa (OMP-1), 34 to 52 kDa (OMP-2), 53 to 97 kDa (OMP-3), and 98+ kDa (OMP-4). Each section was minced, and proteins were eluted using a Model 422 Electro-Eluter (Bio-Rad, Hercules, CA) running at 30 milliamps for 3 h in elution buffer consisting of 25 mM Tris base, 192 mM glycine, and 0.1% SDS. Buffer was then exchanged using Microcon-YM-3 filters (Millipore Corp). Protein concentrations were determined using the Lowry protein assay (30). During some experiments, the lower-molecular-weight fraction of OMPs (OMP-1) was treated with polymyxin B sulfate (30 μg/ml) or proteinase K. OMP-1 was added to proteinase K (10 μg/ml) and incubated at 55°C for 3 h. Proteinase K was inactivated at 100°C for 15 min.

**Statistics.** Significance was determined by a Student's *t* test with two-tailed distribution. *P* values less than 0.05 were considered significant. All experiments were performed in triplicate. Data are presented as the mean ± 1 standard deviation.

## RESULTS

**Induction of MCP-1 gene expression and protein production.** We investigated the effect of *B. henselae* coculture on MCP-1 expression and production in HMEC-1. MCP-1 transcript levels were assayed by semiquantitative RT-PCR at 6 and 24 h after infection (Fig. 1A). HMEC-1 cultured in the presence of *B. henselae* had 1.6 times higher levels of MCP-1 transcript than untreated controls at 6 h after treatment (T6) and 1.8 times higher levels of MCP-1 transcript 24 h after treatment (T24) (Fig. 1A and B). By 48 h after treatment, the MCP-1 message levels for uninfected HMEC-1 began to approach the MCP-1 message levels of *B. henselae*-infected HMEC-1 (data not shown). This was most likely due to the absence of growth factors in culture media; after 48 h of culture with no growth factors, cells lose viability and MCP-1 levels increase.

MCP-1 levels in supernatants of HMEC-1 were analyzed at 6, 24, and 48 h after treatment by ELISA. There was a significant increase in MCP-1 levels when HMEC-1 cells were cultured with *B. henselae* for 6, 24, or 48 h (*P* < 0.007) (Fig. 1C). In order to determine whether the bacterial stimulating molecule was heat stable, bacteria were heat killed by boiling at 100°C for 15 min before addition to HMEC-1. When bacteria were heat killed, MCP-1 levels were not significantly lowered (data not shown). These data indicate that a heat-stable molecule is responsible for MCP-1 production.

**Induction of MCP-1 gene expression and protein production is independent of *B. henselae* LPS and endothelial cell tlr4.** We investigated the role of *B. henselae* LPS in MCP-1 production from HMEC-1. Incubation of HMEC-1 with *B. henselae* LPS did not efficiently induce MCP-1 expression in HMEC-1 (Fig. 2A and B). We also investigated the effect of polymyxin B sulfate, an antibiotic known to neutralize LPS of many bacteria, including *Bartonella* spp. (34). Bacteria (*B. henselae* or *E. coli*) were treated with polymyxin B sulfate before addition to

HMEC-1. Although incubation of *E. coli* with polymyxin B sulfate significantly lowered MCP-1 gene expression and protein production from HMEC-1 at T6, T24, and T48 (*P* ≤ 0.006), polymyxin B had little effect on *B. henselae*-induced MCP-1 expression and production (Fig. 1A, B, and C).

MCP-1 production can be mediated by tlr4 or tlr2 activation (53). Since HMEC-1 express tlr2 poorly (13), we investigated whether *B. henselae*-induced MCP-1 production is mediated through tlr4 on HMEC-1. We preincubated HMEC-1 with a tlr4 mouse monoclonal antibody (HTA125) or an isotype control (mouse IgG2a, κ) and then treated the cells with live *B. henselae* or *E. coli* LPS (1 μg/ml). Supernatants were collected 8 h after infection and assayed for MCP-1 by ELISA. While HTA125 reduced MCP-1 production caused by *E. coli* LPS (*P* < 0.002), *B. henselae*-induced MCP-1 production remained unchanged by the blocking antibody (*P* > 0.900) (Fig. 3A). *E. coli* LPS was used as a positive control for the activity of the blocking antibody; however, the induction of MCP-1 by live *E. coli* JM109 was also lowered in the presence of the blocking antibody (Fig. 3B; *P* < 0.01).

**MCP-1 production requires NF-κB activation.** *B. henselae* outer membrane proteins have been shown to induce NF-κB in HUVEC. Recently, the *virB* (which encodes a putative type IV secretion pathway in *B. henselae*) has also been implicated as a primary factor in NF-κB activation by *B. henselae* in HUVEC (46). In order to determine whether the transcription factor NF-κB was responsible at least partly for MCP-1 production in HMEC-1 cultured with *B. henselae*, NF-κB inhibitors PDTC and TPCK were added to cells before coculture. MCP-1 production in response to both *B. henselae* and *E. coli* LPS (1 μg/ml) decreased significantly in the presence of either inhibitor (*P* < 0.0002) (Fig. 3C).

**Low-molecular-weight outer membrane proteins (OMP-1) from *B. henselae* induce MCP-1 production in HMEC-1.** Since MCP-1 production in response to *B. henselae* appears to occur in an LPS-independent manner, we investigated whether OMPs of the bacteria stimulate MCP-1 production in HMEC-1. It has been shown that OMPs induce NF-κB activation in human umbilical cord vein endothelial cells (HUVEC) (16). Since NF-κB is one of the transcription factors that induce MCP-1 gene expression (42, 55), we investigated whether OMPs could induce production of MCP-1 from HMEC-1. Outer membrane proteins were enriched from *B. henselae* as previously described (6). The OMPs were separated into four molecular weight fractions (see Materials and Methods), and OMPs were added to HMEC-1 culture at 250 ng/ml. Out of the four fractions, only the lowest-molecular-weight fraction, fraction 1 (OMP-1), which corresponds to the OMPs of molecular mass between 3 and 33 kDa, significantly increased MCP-1 production in HMEC-1 at 6 and 24 h after addition of OMPs (*P* < 0.002) (Fig. 4A). OMP-1 also caused MCP-1 production from HMEC-1 in a dose-dependent manner (Fig. 4A). Preincubation of OMP-1 with polymyxin B sulfate did not abrogate its effect (*P* > 0.700) (Fig. 4B), suggesting that endotoxin contamination in the OMP-1 preparation is not responsible for MCP-1 induction. However, proteinase K treatment significantly lowered the effect of OMP-1 (*P* < 0.0002), revealing that the factor in OMP-1 which upregulates MCP-1 production is probably a protein or proteins.
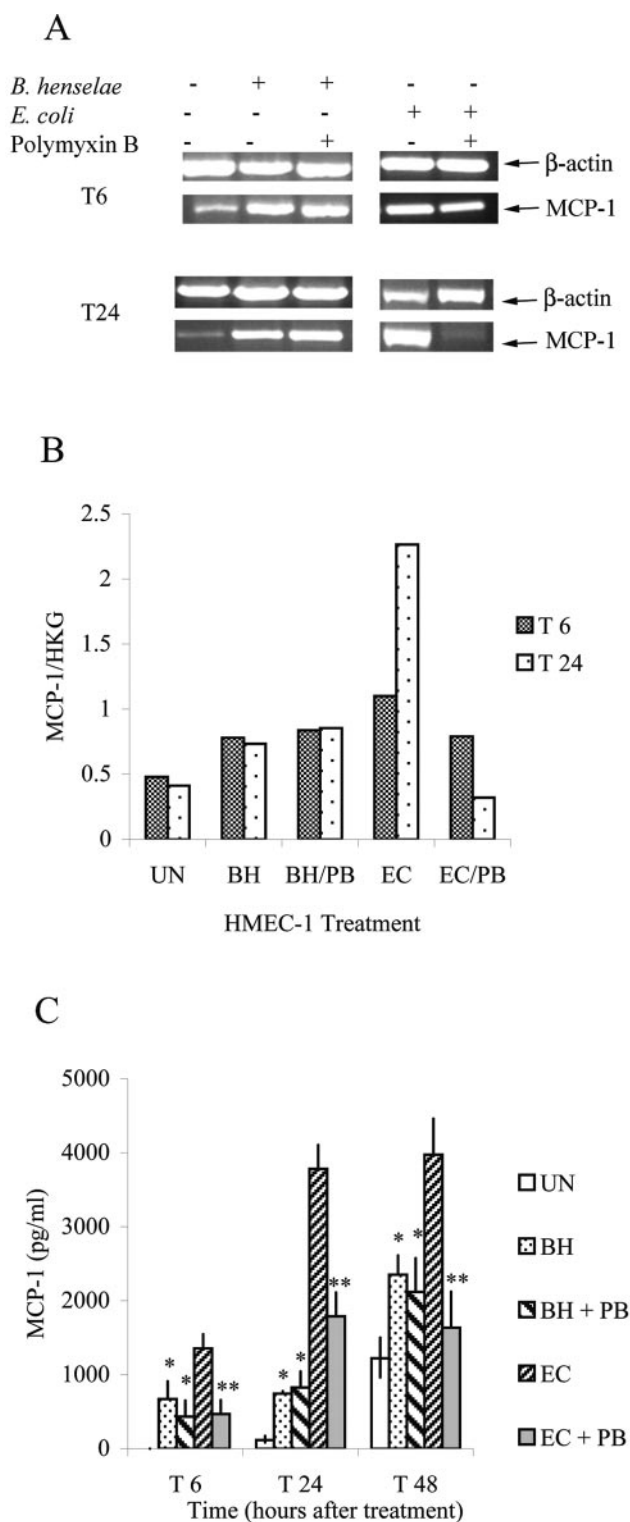
## A



## B



## C



FIG. 1. *B. henselae* induces MCP-1 in HMEC-1. (A) Cells were stimulated with *B. henselae* or *E. coli*. Some bacteria were preincubated with polymyxin B sulfate. The levels of MCP-1 mRNA in HMEC-1 were assayed by RT-PCR at 6 and 24 h after treatment (T6, T24). (B) RNA levels of MCP-1 in cells from experiments whose results are depicted in panel A were determined by scanning densitometry and normalized by comparison to the "housekeeping gene" (HKG) β-actin RNA levels. (C) MCP-1 protein production was determined by ELISA at 6, 24, and 48 h after treatment. (UN = untreated cells; BH = *B.*
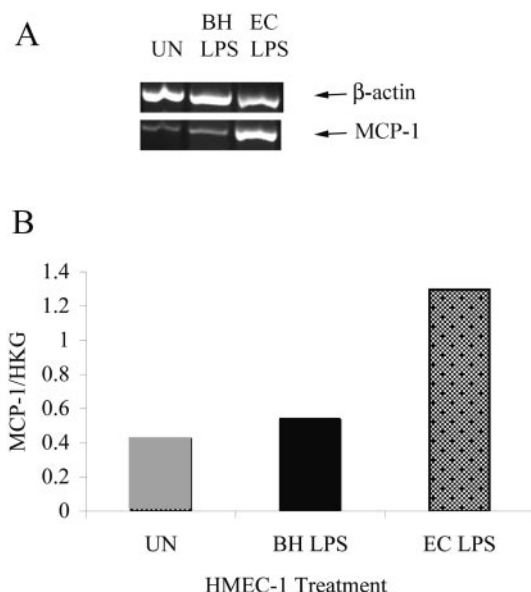
## A



## B



FIG. 2. *B. henselae* LPS does not efficiently upregulate *mcp-1* expression. (A) Cells were stimulated with 1 μg/ml *B. henselae* LPS or *E. coli* LPS. After 8 h, the levels of MCP-1 mRNA were assayed by RT-PCR. (B) RNA levels of MCP-1 in cells from experiments whose results are depicted in panel A were determined by scanning densitometry and normalized by comparison to housekeeping gene (HKG) β-actin levels. UN = untreated cells; BH = *B. henselae*-treated cells; EC = *E. coli*-stimulated cells.

**Supernatants from *B. henselae*-treated HMEC-1 induce chemotaxis of THP-1 monocytes.** Since MCP-1 levels are elevated in response to *B. henselae*, we investigated whether supernatants from *B. henselae*-treated HMEC-1 could cause chemotactic migration of THP-1 monocytes. A modified Boyden chamber assay was used to assess the chemotactic response of THP-1 monocytes to chemoattractants present in the supernatant from *B. henselae*-treated HMEC-1. Migrated cells were fixed, stained, and counted in five high-powered fields. Supernatants from untreated HMEC-1 were used as negative controls, and supernatants from *E. coli*-treated HMEC-1 were used as positive controls. Supernatants from *B. henselae*-infected HMEC-1 induced THP-1 chemotaxis at a level at least 6 times that of supernatants from uninfected HMEC-1 (Fig. 5) and at levels similar to those of supernatants from *E. coli*-infected HMEC-1 (4 times that of uninfected supernatants).

## DISCUSSION

*B. henselae* causes disease characterized by vascular proliferative lesions (3) into which macrophages infiltrate during infection (26, 27, 35). In the paracrine loop model of angiogenesis, macrophages are implicated as effector cells; upon stimulation by *B. henselae*, they secrete VEGF and other en-

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*henselae*-treated cells; PB = polymyxin B sulfate; EC = *E. coli*-stimulated cells; * = $P < 0.007$ [*B. henselae* treatment compared to untreated cells]; ** = $P < 0.007$ [untreated *E. coli* compared to *E. coli* treated with polymyxin B sulfate]). Results shown are those of one experiment representative of three similar experiments.
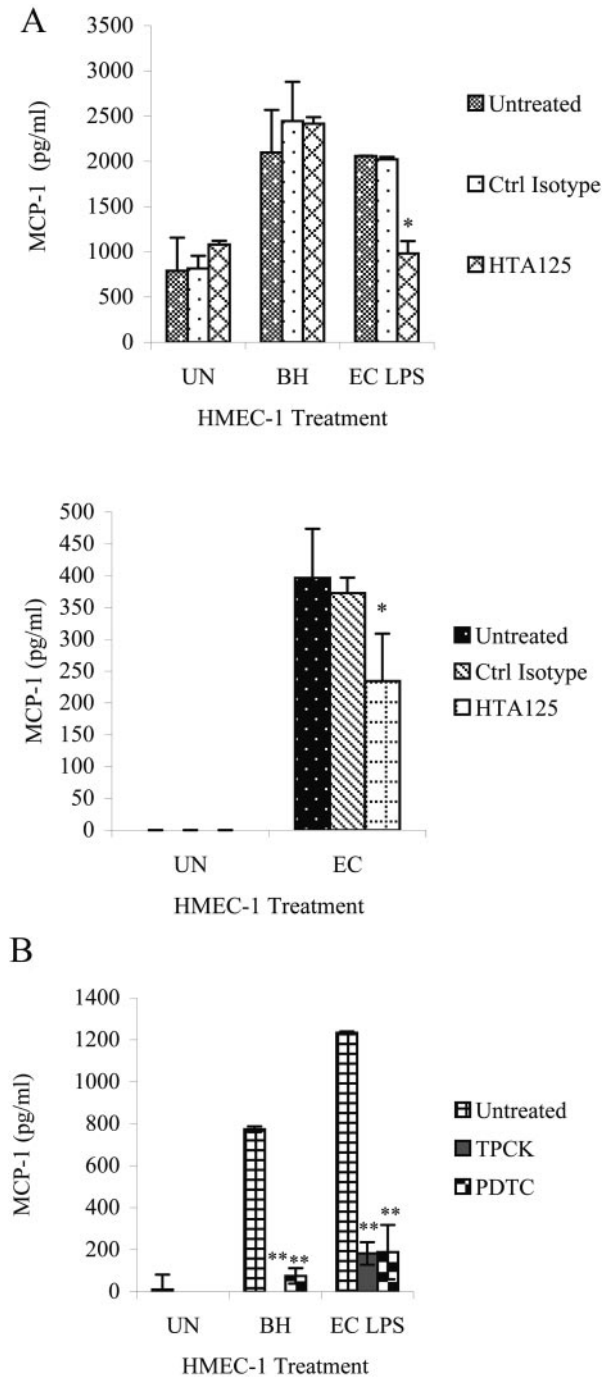
FIG. 3. MCP-1 production during inhibition assays. Supernatants were collected 8 h after infection and analyzed by ELISA for MCP-1 levels. (A) Cells were incubated with anti-tlr4 (HTA125) or a control isotype (mouse IgG2a, κ) for 1 h before coculture with *B. henselae* or addition of *E. coli* LPS (1 μg/ml) (upper panel). Cells were incubated with HTA125 or a control isotype for 1 h before addition of live *E. coli* JM109 (lower panel). (B) HMEC-1 cells were cultured in the presence of the NF-κB inhibitor PDTC or TPCK before infection with *B. henselae* or addition of *E. coli* LPS (1 μg/ml). (UN = untreated HMEC-1; BH = HMEC-1 cocultured with *B. henselae*; EC LPS = HMEC-1 stimulated with *E. coli* LPS; EC = HMEC-1 stimulated with live *E. coli*; * = $P < 0.05$; ** = $P < 2.00 \times 10^{-4}$). A representative of three experiments is shown.
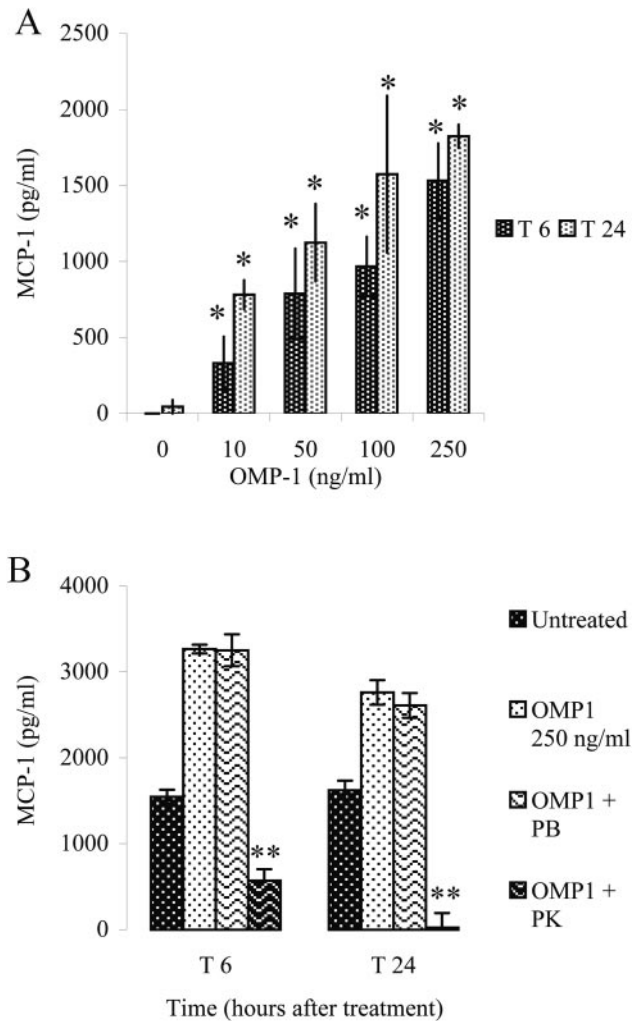


FIG. 4. MCP-1 production in response to OMP fraction 1. HMEC-1 cells were stimulated with OMP-1 with various dosages and under various conditions. Supernatants were collected at 6 and 24 (T6, T24) h after addition of OMP-1. MCP-1 levels were determined by ELISA. (A) Dose-dependent response of HMEC-1 to various OMP-1 concentrations. (B) Effects of polymyxin B sulfate (30 μg/ml) (PB) and proteinase K (10 μg/ml) (PK) on OMP-1-induced MCP-1 production in HMEC-1 cells. (* = $P < 0.002$; ** = $P < 2.00 \times 10^{-4}$). A representative of three experiments is shown.

dothelial cell mitogens (41). Concurrently, endothelial cells upregulate pro-angiogenic factors such as chemokines, inhibit apoptosis through inhibition of caspases (22), and upregulate adhesion molecules (16) which may promote proliferation. In this study we investigated the expression and production of the chemokine MCP-1 from *B. henselae*-stimulated HMEC-1. MCP-1 is a member of the C-C chemokine family and is produced and secreted by monocytes, fibroblasts, and vascular endothelial cells. MCP-1 then interacts with its CCR2B receptor on monocytes and macrophages to cause chemotaxis (8). MCP-1 can also directly promote angiogenesis. When tumor cells are transfected with the *mcp-1* gene and injected into a murine model, angiogenesis is stimulated (37). In addition, MCP-1 implants induce angiogenesis in a rabbit cornea (52). During *B. henselae* infection, MCP-1 released from endothelial
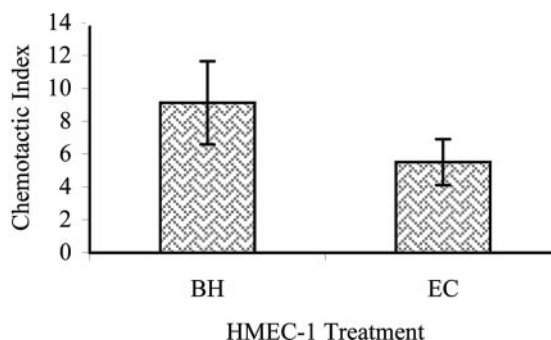
FIG. 5. THP-1 monocyte chemotaxis in response to supernatants from HMEC-1. Chemotaxis of THP-1 monocytes in response to supernatants from untreated HMEC-1, supernatants from HMEC-1 cultured with *B. henselae* (BH), and supernatants from HMEC-1 treated with live *E. coli* (EC). Results are expressed as a chemotactic index. The number of migrated THP-1 in five microscopic fields was averaged for supernatants from untreated, *B. henselae*-treated, and *E. coli*-treated HMEC-1. The chemotactic index for cells which migrated in response to uninfected supernatants was set at 1, and other samples were normalized accordingly. Results shown are the means of three separate experiments.

cells can cause chemotaxis of monocytes and macrophages to the site of infection, thereby promoting an angiogenic state by recruiting the effector cell.

Bacterial pathogens such as *E. coli*, *Orientia tsutsugamushi*, and *Porphyromonas gingivalis* increase the production and secretion of chemokines such as IL-8 and MCP-1 (9, 24, 59). MCP-1 is induced in HMEC-1 in response to *B. henselae*. Both mRNA and protein levels are upregulated; mRNA levels are higher than those of untreated controls in *B. henselae*-treated HMEC-1 at 6 and 24 h posttreatment, while protein levels in treated cells are higher at 6, 24, and 48 h posttreatment. Furthermore, supernatants from *B. henselae*-stimulated HMEC-1 caused chemotaxis of THP-1 monocytes. Thus, the levels of MCP-1 produced by HMEC-1 in response to *B. henselae* in vitro are sufficient to function as a chemoattractant for monocytes. Results also reveal that the bacterial factor which causes MCP-1 production is probably a heat-stable molecule.

The lipopolysaccharide of *B. henselae* has recently been characterized as containing a lipid A possessing features known to reduce endotoxicity, including a pentaacyl lipid A and a long-chain fatty acid (58). *B. henselae* LPS induces tlr4 levels 1,000-fold lower than *Salmonella enterica* sv. Friedenau LPS (58). In addition, LPS from *B. quintana*, which is likely quite similar to *B. henselae* LPS, induces IL-8 (GRO-CINC-1 in rats) but not TNF in rats or human whole blood (34). *B. henselae* LPS also does not induce TNF in cats (34). In this study, the addition of polymyxin B sulfate to *B. henselae* before addition to HMEC-1 did not reduce MCP-1 production; however, polymyxin B sulfate had a significant lowering effect on *E. coli*-induced MCP-1 production. Moreover, the LPS from *B. henselae* does not induce upregulation of MCP-1 expression in HMEC-1. These data corroborate with the low endotoxicity of LPS from *Bartonella* spp. to imply a limited or nonexistent role for LPS in *B. henselae*-induced MCP-1 production.

Toll-like receptors activated by various microbial products can cause expression and production of chemokines (11, 12, 31), including MCP-1 (45). LPS, a tlr4 agonist, causes MCP-1

production in a tlr4-dependent manner (53). Most studies confirm that MCP-1 production is tlr4 mediated and usually caused by LPS in a bacterial infection. However, recently it was discovered that tlr4-deficient and tlr4-competent mice have the same MCP-1 response to infection by *Leishmania major* (4), which is known to cause chemokine production early in infection (20). Our findings indicate that MCP-1 production in response to *B. henselae* is not tlr4 dependent. In contrast, *E. coli* LPS-induced MCP-1 production was lowered in the presence of a tlr4 monoclonal antibody. These data suggest the possibility of an alternate pathway to tlr4 activation for the MCP-1 production from *B. henselae*-treated HMEC-1. Furthermore, these results again exclude *B. henselae* LPS from a role in MCP-1 production. HMEC-1 express tlr1, tlr3, tlr4, and tlr5 but express tlr2 very weakly, which is why they are unresponsive to tlr2 ligands (13). Thus, the MCP-1 production investigated in this study is probably not tlr4 or tlr2 mediated. Other tlr or similar receptor pathways must be investigated to pinpoint the exact mechanism of MCP-1 induction in HMEC-1 in response to *B. henselae*.

The *mcp-1* gene contains binding sites for both NF-κB and AP-1 (42, 55), and both transcription factors have been implicated in *mcp-1* expression (9, 57). It has been established that *B. henselae* induces NF-κB-dependent upregulation of adhesion molecules in HUVEC independent of LPS (16). In this study, we used two NF-κB inhibitors to determine whether MCP-1 protein production requires NF-κB activation. Diverse NF-κB inhibitors have been used with HMEC-1 previously in similar experiments (9). PDTC is an antioxidant that inhibits the phosphorylation of IκB (36, 47), and TPCK inhibits proteosome-dependent degradation of inhibitory peptides (32). Consequently, through the use of these inhibitors, we demonstrated that MCP-1 production caused by *B. henselae* in HMEC-1 is NF-κB dependent.

OMPs of *B. henselae* are important for pathogenesis (6, 16). OMPs have been implicated in the NF-κB-dependent upregulation of adhesion molecules in HUVEC (16). However, it has also been determined that NF-κB-dependent proinflammatory response to *B. henselae* in HUVEC is mediated primarily by *virB* (46), the operon which encodes a putative type IV secretion system in *B. henselae*, indicating a role for secreted and transported proteins in NF-κB activation. However, data presented here reveal the ability of *B. henselae* Houston-1 OMPs, specifically OMPs of molecular mass between 3 and 33 kDa, to enhance production of the C-C chemokine MCP-1 from HMEC-1. We have also shown that the MCP-1 induction seen here is NF-κB dependent through the use of two NF-κB inhibitors. Thus, bacterial-induced NF-κB upregulation may occur from a combination of mechanisms. Incubation of OMP-1 with polymyxin B sulfate before addition to HMEC-1 reveals that this MCP-1 induction is LPS independent. These data point to a heat-stable low-molecular-weight OMP of *B. henselae* Houston-1 that contributes at least in part to *B. henselae*-induced MCP-1 production from endothelial cells. Further studies are needed in order to specify the putative OMP that causes MCP-1 upregulation in endothelial cells.

We have described upregulation of gene expression and protein production of the chemokine MCP-1 in response to *B. henselae*. This stimulation of HMEC-1 is independent of *B. henselae* LPS and toll-like receptor 4 but dependent on NF-κB

activity. MCP-1 produced by infected HMEC-1 most likely contributes to the ability of conditioned media from these cells to induce monocyte chemotaxis. The recruitment of macrophages by MCP-1 produced from infected endothelial cells could have broad implications on mechanisms of angiogenesis during this infection. Specifically, the macrophage effector cell which secretes VEGF and other angiogenic factors is brought to the site of infection. Pathogenic angiogenesis provides actively growing target cells for *B. henselae* in an enriched vascularized microenvironment, and while the specific role of MCP-1 induction in this phenomenon is not completely understood, we suggest that recruitment of the monocyte/macrophage effector cell is an important component of the pathway.

## ACKNOWLEDGMENTS

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